



# MicroRNA miR-125a Controls Hematopoietic Stem Cell Number

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# **microRNA-125a controls hematopoietic stem cell number**

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MicroRNAs influence hematopoietic differentiation, but little is known about their effects on the stem cell state. Here, we report that the microRNA processing enzyme Dicer is essential for stem cell persistence *in vivo* and a specific microRNA, miR-125a controls the size of the stem cell population by regulating stem/progenitor cell (HSPC) apoptosis. Conditional deletion of Dicer revealed an absolute dependence for the multipotent HSPC population in a cell autonomous manner, with increased HSPC apoptosis in mutant animals. An evolutionarily conserved microRNA cluster containing miR-99b, let-7e and miR-125a was preferentially expressed in long term HSCs. miR-125a alone was capable of increasing the number of hematopoietic stem cells *in vivo* by more than eight fold. This was accomplished through a differentiation stage-specific reduction of apoptosis in immature hematopoietic progenitors, possibly through targeting multiple pro-apoptotic genes. Bak1 was directly down-regulated by miR-125a and expression of a 3'UTR-less Bak1 blocked miR-125a-induced hematopoietic expansion *in vivo*. These data demonstrate cell-state-specific regulation by microRNA and identify a unique microRNA functioning to regulate the stem cell pool size.

body Hematopoietic stem cells (HSC) self-renew and differentiate to form all blood cells throughout animal life. The intricate balance between these two characteristic stem cell states is required for maintaining hematopoietic homeostasis and responding to tissue injury. Stem cell population size is tightly regulated and thought to be dictated by rates of proliferation, relative frequency of differentiative versus self-renewal outcomes and apoptosis. Disruption of any of these processes could lead to stem cell exhaustion or increased risk of leukemogenesis (1-5). However, the molecular events specifying stem cell population size are still poorly understood.

MicroRNAs are emerging as a class of important cellular regulators that mediate cell state with specific patterns of microRNA expression demarcating developmental or differentiation stages (6-8). They are transcribed as longer primary microRNAs and their maturation is dependent on the RNase III enzyme, Dicer (9-13). In the blood system, multiple microRNAs have been found to direct differentiation, e.g. miR-181 for T cells (14), miR-150 for B cells (15, 16) and miR-223 for granulocytes (17-19). We have shown that miR-150, shunts megakaryocyte and erythrocyte common progenitors (MEP) toward megakaryocytes (20). To date, all known microRNAs reinforce specific lineage outcome and no specific microRNAs are known to regulate the number of stem/progenitor cells in the hematopoietic system.

## RESULTS

### **Hematopoietic ablation of *Dicer* impaired the hematopoietic stem/progenitor compartment**

We hypothesized that microRNAs regulate HSCs and first evaluated this using a mouse with a conditional allele of the microRNA processing enzyme Dicer (10, 13). *Dicer*<sup>lox/lox</sup> mice were bred with MxCre mice, which express the Cre recombinase in response to

interferons (IFN) and can be experimentally induced with high efficiency in blood cells, including HSCs, via peritoneal injection of polyI:polyC (plpC) (4, 5, 21). Mice with the genotypes of *Cre<sup>+</sup>Dicer<sup>lox/lox</sup>* (mutant) and *Cre<sup>+</sup>Dicer<sup>wt/wt</sup>* or *Cre<sup>+</sup>Dicer<sup>lox/wt</sup>* littermates (control) were used as we did not observe differences between *Cre<sup>+</sup>Dicer<sup>wt/wt</sup>* and *Cre<sup>+</sup>Dicer<sup>lox/wt</sup>* mice.

HSC alteration by *Dicer* loss was assessed by long-term repopulation, a definitive assay for HSCs. Whole bone marrow (BM) from control or mutant mice (CD45.2+) prior to plpC treatment were mixed 1:1 with wild-type competitor BM (CD45.1+) and transplanted into lethally irradiated recipient mice (CD45.1+). Seven doses of plpC were administered 5 weeks after transplantation given every other day over a course of 13 days. The day of the last plpC injection was counted as day 0. The contribution to T, B and myeloid lineages in the peripheral blood (PB) was monitored over time (Figure 1A and B, Figure S1). While both mutant and control groups showed ~50% overall donor type (CD45.2+) reconstitution before plpC injection, reconstitution by mutant marrow markedly declined after plpC treatment, and remained reduced until 20 weeks post-plpC, when donor contribution was primarily stem-cell-derived. The reduction in reconstitution by mutant BM could also be observed in secondary transplant recipients (Figure S1C), underscoring the importance of *Dicer* in HSCs.

Two lines of evidence support the notion that HSPCs are impaired by *Dicer* loss, rather than the alternative possibility that the reduction in mutant marrow repopulation capacity is completely due to impairing multiple independent committed lineages. First, mutant marrow gave reduced donor cell contribution to the Lin<sup>-</sup>Kit<sup>+</sup>Sca<sup>+</sup> (LKS) population (Figure 1C), which contains all HSCs and multipotent progenitors. In addition, this reduction paralleled in magnitude the reduction in committed cell types. Second, the decline of mutant BM reconstitution occurred progressively after plpC treatment over a period of 8-10 weeks (Figure 1A). Importantly, the drop in myeloid reconstitution was

evident as early as 1 day after the last dose of plpC (13 days after initiation of plpC), while the lymphoid lineages were still intact at this time (Figure S1D). This is consistent with the difference in the turnover rate among these lineages, with the rapidly turning-over myeloid cells reflecting damage in the immature hematopoietic pools earlier than the longer-lived lymphoid populations. While we regard these data as consistent with an effect on more primitive cells, we cannot exclude and do not assert that *Dicer* deletion does not have effects on lineage committed cells.

Donor cells were present months after plpC treatment with largely normal lineage distribution (Figure S1A) raising the possibility that *Dicer* was not essential for HSPC function or that some HSCs had escaped Cre mediated *Dicer* excision. To evaluate this, we sorted donor cells from the PB 6 months post transplant (Figure S2A). Analyses of the genomic DNA indicate that all mice transplanted with control *Cre*<sup>+</sup>*Dicer*<sup>lox/wt</sup> cells showed complete deletion of the loxed alleles, however, in mice that received mutant *Cre*<sup>+</sup>*Dicer*<sup>lox/lox</sup> cells, the loxed allele (functionally wild type allele) persisted (Figure S2A, top table). To test deletion at a clonal level, donor-type (CD45.2+) BM cells were sorted and plated into methylcellulose. No colonies could be identified with a *Dicer*<sup>Δ/Δ</sup> genotype (0/34) (Figure S2A, bottom table). In contrast, the control *Dicer*<sup>lox/wt</sup> colonies all showed a *Dicer*<sup>Δ/wt</sup> genotype (38/38). *Dicer*<sup>Δ/Δ</sup> colonies were also completely absent from BM cells of un-manipulated mutant animals following plpC injections, whereas control *Dicer*<sup>lox/wt</sup> BM colonies again displayed 100% deletion of the loxed allele (Figure S2B). These data support that homozygous *Dicer* deletion is incompatible with a functional HSC state.

To evaluate the basis for cell loss, apoptosis was assayed immediately following plpC treatment in otherwise un-manipulated mice. LKS cells, Lin<sup>-</sup>Kit<sup>+</sup>Sca<sup>-</sup> (L-K+S-) cells (containing myeloid progenitors) and another heterogeneous population Lin<sup>-</sup>Kit<sup>+</sup>Sca<sup>+</sup> (L-K+S+) (22, 23) were examined for caspase-3 activation. Mutant LKS cells displayed a consistent and significant increase in apoptosis, whereas the L-K+S- myeloid progenitors

and L-K-S+ population were less affected (Figure 1D and S3A). Meanwhile, mutant BM demonstrated increased Ki67 staining, suggesting a compensatory response to cell loss (Figure S3B, C) following plpC treatment. Similar results were observed when *Dicer* deletion was induced by IFN $\beta$  in FACS purified HSPCs *in vitro* (Figure S4).

The notion that *Dicer* loss causes apoptosis in HSPCs predicts that stem cell niche could be vacated to allow engraftment of exogenous stem cells. We tested this possibility by transplanting  $5 \times 10^6$  wild-type BM (CD45.2+) mononuclear cells into control or mutant animals (CD45.1+) following 7 doses of plpC, but without irradiation (Figure S3D). Indeed, minimal engraftment was observed in control mice as would be expected from the few available stem cell niches at homeostasis. In contrast, robust CD45.2+ donor engraftment was observed in mutant mice, with contribution to both myeloid and lymphoid lineages five months post-transplantation (Figure S3D, red gate). These data further support HSPC impairment by *Dicer* loss and suggest HSC death caused by *Dicer* deficiency.

Taken together, the data above indicate that *Dicer* is necessary for HSPCs and its loss compromises HSPC function in a manner consistent with stem cell death.

### **A microRNA cluster preferentially expressed in LT-HSCs**

Since microRNAs are major substrates for *Dicer* and microRNAs specify cellular states, we hypothesized that specific microRNAs contribute to the functional maintenance of a HSC state, as defined by persistent self-renewal capability coupled with multilineage differentiation capacity. To identify such microRNAs, we performed microRNA expression profiling in multiple stem cell and progenitor populations with well-defined markers using a bead-based expression analysis platform (6, 20) followed by RT-PCR validation (Figure 2A and B, Figure S5A). Interestingly, hierarchical clustering indicates that microRNA expression profiles reflect self-renewal and multipotency, with the more



primitive populations clustered closely (Figure 2A). Specifically, we found multiple microRNAs preferentially expressed in populations that self-renew (Table S1). Of particular interest, three microRNAs, miR-99b, let-7e and miR-125a, were highly expressed in long-term (LT) HSCs compared to other populations (Figure 2B and S5A). These three microRNAs display a complete evolutionary conservation among mammals and organize in a cluster spanning a ~600 bp region on chromosome 19 in human and 17 in mice (Figure S5B).

We tested the function of the miR-99b-let-7e-miR-125a cluster in regulating HSCs using retroviral expression (vector also expresses GFP) followed by transplantation (20). BM cells expressing the microRNA cluster showed enhanced reconstitution in all major blood lineages after long-term transplantation (Figure 2C and D). In contrast, reconstitution by control-vector-transduced cells declined over time (Figure 2C). The increase in multi-lineage reconstitution by the miR-99b-let-7e-miR-125 cluster persisted in secondary transplantation (Figure S6A), consistent with an effect on HSCs. This increase in reconstitution was not due to a difference in transduction efficiency of the cluster versus control vectors, as both were similar immediately post-transduction (Figure 2C).

### **A single microRNA, miR-125a, augmented HSC activity**

Individual microRNAs in the cluster were then analyzed. MiR-125a alone, but not miR-99b or let-7e, provided comparable increase in long-term multi-lineage reconstitution (Figure 3A, B, C and D). Remarkably, this enhancement appears to occur in the absence of a specialized BM microenvironment or niche, as 18-day *ex vivo* culture in the presence of miR-125a preserved robust stem cell activity, which was completely lost in the control cultures (Figure 3E and S6B).

We next sought to determine if endogenous miR-125a regulates primitive hematopoietic cell function. To this end, we used antagomir against miR-125a, a chemically synthesized cell-permeable microRNA inhibitor (24). This sequence-specific inhibitor of miR-125a did not affect methylcellulose colony morphology or numbers in primary cultures, but drastically reduced colony formation in subsequent serial replating cultures (Figure 3F). Since this assay is an *in vitro* surrogate for self-renewal, these data are consistent with miR-125a regulation of HSPC self-renewal.

### **MiR-125a amplifies the HSC pool size**

To more precisely quantify the effect of miR-125a on stem cells, we performed the competitive limiting dilution assay (LDA). The need for this functional assay was substantiated by the observation that conventional stem cell surface markers were markedly changed with miR-125a ectopic expression, precluding accurate immunophenotypic enumeration. LDA analysis was performed using total GFP<sup>+</sup> BM cells from recipients after 4-5 months in primary transplantation (Table 1). The number of myeloid cells predominated with a reduced proportion of lymphoid cells (Table S2 and Figure 3D). We scored both myeloid (Mac1<sup>+</sup>) and lymphoid lineages (B220<sup>+</sup> or CD3<sup>+</sup>) in our LDA with  $\geq 1\%$  as cutoff. Although the apparent frequency of reconstituting HSCs varied between experiments (presumably due to different extent of HSC exhaustion under this experimental setting), we could consistently detect >8 fold expansion of the reconstituting HSC pool (Table 1).

### **MiR-125a-induced stem cell amplification is cell-stage-specific**

We next sought to determine if miR-125a expression confers self-renewal capacity regardless of the ground cell state. Purified common lymphoid progenitors (CLP), common myeloid progenitors (CMP), granulocyte and macrophage progenitors (GMP)

and megakaryocyte and erythrocyte progenitors (MEP), as well as the LKS cells were tested for their ability to gain or maintain the stem cell property of self-renewal with forced expression of miR-125a (Figure 3G). The production of mature cells in the PB from CLP, CMP, GMP and MEP was minimal and disappeared completely in the early weeks (3-6 weeks) post-transplantation. In contrast, LKS cells transduced with miR-125a reconstituted >70% of PB starting from 3 weeks post-transplantation and lasted 5 months (Figure 3G) and through secondary transplantation (see limiting dilution assays Table 1 and S2). Similar results were obtained with further purified HSCs (LKSCD48-CD150+) as the starting population (Figure 3H). These results indicate that miR-125a was insufficient to induce self-renewal in committed progenitors; its effect depends upon the underlying cell state.

#### **MiR-125a protected primitive hematopoietic cells from apoptosis**

Lastly, since Dicer loss induced apoptosis in HSPCs, we asked whether expression of miR-125a may protect primitive cells from apoptosis. BM from mice transplanted with control- or miR-125a-transduced cells were analyzed for apoptosis after re-establishment of homeostasis. We consistently observed decreased apoptosis in miR-125a-transduced cells in the lineage-negative population, but not in the more mature lineage-positive cells (Figure 4A-B), indicating a cell-type-specific effect of miR-125a. These data are consistent with the anti-apoptotic effect being at least partially responsible for the increase in the reconstituting cell pool.

#### **MiR-125a regulates the pro-apoptotic protein Bak1**

Given the reduced apoptosis in primitive cells and the greatly expanded HSC pool size, we reasoned that miR-125a could target pro-apoptotic proteins to tilt the cellular balance of pro- and anti-apoptotic signals. miR-125a is predicted to target over 500 evolutionarily

conserved targets (25), a number of which have been reported with roles in apoptosis either directly or indirectly. We examined the pro-apoptotic protein, Bak1 (Bcl-2 antagonist/killer1). HL-60 or BaF3 cells were transduced with miR-125a or the control vector. In both cases, miR-125a expression reduced endogenous Bak1 protein by ~40-50% (Figure 4C and S7A). Scanning the 3' untranslated region (UTR) of Bak1 revealed one conserved miR-125a targeting site (Figure S5C). To ascertain whether the inhibitory effect of miR-125a was mediated through the specific target site in its 3'UTR, we fused the UTR sequence to a luciferase reporter. MiR-125a caused ~50% inhibition of the luciferase activity. In addition, mutation of the conserved targeting site alleviated most of the inhibition by miR-125a (Figure 4D). Consistent with the observation that miR-125a is the single microRNA within the miR-99b-let-7e-miR-125a cluster that mediates HSC expansion, miR-125a inhibited the Bak1 3'UTR construct (Figure S7B), whereas miR-99b and let-7e had minimal effect.

We next asked whether forced expression of Bak1 could block miR-125a-mediated hematopoietic expansion. We co-expressed miR-125a with either control or Bak1 in donor marrow (Figure 4E-F and S8). Co-expression events were marked with DsRed-Express and GFP double positive cells as miR-125a and Bak1 are expressed from vectors that also carry these two fluorescent proteins respectively. The percentages of all three populations increased during 6 days of *in vitro* culture. Although the increase for cells expressing Bak1 was always slower, Bak1 expressing cells were clearly detectable at all times and therefore the levels of Bak1 were not simply eliminating transduced cells (Figure 4E and S8). Upon transplantation, miR-125a and control vector co-transduced marrow expanded as expected (Figure 4F). In contrast, donor marrow co-expressing miR-125a and Bak1 failed to contribute to PB at detectable levels, despite similar percentages of double positive cells 2 days after infection (1.12% and 1.02% for control and Bak1, respectively. Figure 4E). This data demonstrate that sustained Bak1

expression blocks miR-125a-induced hematopoietic expansion. To evaluate whether blocking Bak1 mimics the effect of miR-125a, we examined HSCs in mice engineered to be deficient in Bak1 (26, 27). We did not observe significant alteration in phenotypic and functional HSCs from Bak1<sup>-/-</sup> marrows, suggesting either that miR-125a achieves hematopoietic expansion through targeting additional targets simultaneously (see discussion) or that the compensatory expression of Bak1 family members in the constitutive knock-out may obscure a more potent effect of Bak1.

Taken together, our data indicate that miR-125a protects HSPCs from apoptosis and promote extensive expansion of the hematopoietic stem cell pool.

## DISCUSSION

We report an essential role of *Dicer* for HSPC maintenance and the identification of a single microRNA, miR-125a, capable of positively regulating HSC regeneration of hematopoiesis at least in part by reducing apoptosis. We provided multiple lines of evidence indicating HSPC impairment and increased apoptosis induced by *Dicer* deficiency, but our data do not exclude that *Dicer* loss may also impair more differentiated cells or the differentiation process itself (12, 15, 28, 29). Further, they cannot exclude that some of the impact of *Dicer* deletion may be augmented by the conditions under which we deleted that gene. The use of plpC intentionally induces interferons and recent findings indicate that interferon signaling modulates HSCs (30, 31), it is thus possible that plpC-activated HSPCs are more susceptible to the deleterious effect of *Dicer* loss.

The basis for the *Dicer* effect on HSPC is thought to be its role in microRNA processing. To that end, we identified miR-125a to amplify HSC number and protect primitive cells against apoptosis. Apoptosis modulation has been shown to alter HSCs *in vivo* with either forced Bcl-2 expression (32, 33) or MCL-1 deletion (34). We show that

miR-125a has a differentiation stage specific effect increasing the relative abundance of cells in the stem cell state by preventing their apoptosis. The anti-apoptotic effect of miR-125a is associated with its ability to down-regulate a pro-apoptotic protein Bak1. Bak1 is a direct target of miR-125a. We did not observe altered HSC number/activity in Bak1<sup>-/-</sup> mice. These data could be due to compensation by other Bak1 relatives in the constitutive knock-out or that additional direct or indirect pro-apoptotic protein targets are required for the phenotype induced by miR-125a. Given that miRNAs generally have multiple targets (25), it is certainly possible that their effect depends upon the combinatorial action of several molecules. Our data merely support that Bak1 is likely to be one of those molecules. This notion is supported by the fact that Bak1<sup>-/-</sup>Bax<sup>-/-</sup> mice displayed increases in both myeloid and lymphoid lineages (27). Indeed, we observed Puma (Bbc3) protein down-regulation by miR-125a in BaF3 cells, whereas BMF is a target for a miR-125a family member (35). While it is technically difficult to mimic the down-regulation of multiple anti-apoptotic proteins in HSCs simultaneously, we demonstrate that sustained Bak1 expression ablates the ability of miR-125a to induce hematopoietic expansion, supporting a role of the apoptotic pathway in mediating the effect of miR-125a. Recently, human p53 has been reported to be targeted by miR-125b (36), a homolog of miR-125a. However, the targeting site identified in this study is not conserved in mouse, consistent with our observation of a lack of significant effect of miR-125a on mouse p53 3'UTR reporter. Hence p53 is an unlikely candidate that accounts for the superior HSC expansion seen with miR-125a.

In addition to the effect on stem cells and the amplification of all major lineages, we also noticed that sustained expression of miR-125a skewed lineage distribution, favoring the myeloid fate and compromising the B lymphoid fate (Figure 3D and Table S2). Although the endogenous level of miR-125a is high in LT HSCs and much lower in progenitors, we do not exclude a role for miR-125a in committed hematopoietic

progenitors, during lineage commitment or in more mature blood cells. Since HSCs are heterogeneous, it is also possible that miR-125a may have selectively expanded a more myelogenic subtype (designated  $\alpha$  type HSC by Dykstra et al. (37)) or influenced other subtypes to be more  $\alpha$ -like. We also note that miR-125b has been reported to be involved in leukemic translocation suggesting the possibility that this microRNA can participate in malignant hematopoiesis (38), although the mechanism and cell of origin remain to be investigated.

In summary, we report that microRNAs are actively participating in regulating the HSC state with sensitivity of HSPCs to the loss of the microRNA processing enzyme Dicer and with the unique capability to have HSC number increased by a single microRNA, miR-125a. The ground state of the cell affects its response to microRNAs and suggest that microRNA-based cell modification may be a means to achieve stem cell specific therapeutics.

## **MATERIALS AND METHODS**

The Subcommittee on Research Animal Care of the Massachusetts General Hospital approved all animal work. The *Dicer*<sup>lox/lox</sup> mice were described before (28). All other mice were purchased from the Jackson Laboratory. Methylcellulose M3434 (StemCell Technologies) were used for colony forming assays. Luciferase reporter assay was performed as described (20). MicroRNA expression constructs were cloned into pMIRWAY-GFP as described (20). Alternatively, GFP was replaced with DsRed-Express (Clontech). For protein expression, the Bak1 open reading frame (ORF) was purchased from Invitrogen and subcloned into the pMIRWAY-GFP vector. Viral production, infection and bone marrow transplantation were performed as described (20). Except where specified, all data are mean  $\pm$  SD. *p* values were calculated using 2 tailed, unequal

variance Student *t* test. More details for materials and methods are included in the Supporting Information.

## Supporting Information

The Supporting Information includes eight figures and two tables. MicroRNA expression data have been deposited to GEO database.

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## FIGURE LEGENDS

### Figure 1. *Dicer* Deletion Abolishes Functional and Immuno-phenotypic HSPCs.

**(A)** Peripheral blood chimerism by control and mutant BM in a 1:1 competitive transplantation assay. The 7 arrows indicate plpC injections. Each dot on the line indicates the average donor-type cell percentage (%CD45.2+) at the indicated time points (d: days; w: weeks after plpC injection). n = 15. **(B)** Lineage contribution by donor-type cells 20 weeks after plpC injections. Lineages analyzed include myeloid (Mac-1+), B (B220+) and T cells (CD3+). Error bars indicate standard deviation. \* p < 0.05. **(C)** Representative FACS plot showing donor-type LKS cells in recipient BM 6 months post plpC injections. **(D)** Intra-cellular flow cytometry for activated caspase-3 in three BM populations including the Lin-c-Kit+Sca+ (LKS), Lin-c-Kit+Sca- (L-K+S-) and Lin-c-Kit-Sca+ (L-K-S+) cells.

### Figure 2. MiR-99b-let-7e-miR-125a Cluster Expression Enhances Long-term Multi-lineage Reconstitution.

**(A)** Heatmap of microRNA expression profiles of hematopoietic cells. Population designation: Sca+ = Lin-c-Kit-Sca+, BM = whole BM, K = Lin-c-Kit+Sca-, LKS = Lin-c-Kit+Sca+, LT = Lin-c-Kit+Sca+CD34-Flk2-, MPP = Lin-c-Kit+Sca+CD34+Flk2+ and ST = Lin-c-Kit+Sca+CD34-Flk2+. Each column represents an independent sample. Populations were sorted from pooled BM cells from multiple animals on multiple days. Red color indicates higher expression; blue for lower. **(B)** Bar graph of data in (A) for miR-125a, miR-99b and let-7e. **(C)** 5FU primed wild type donor marrow was transduced with retrovirus expressing either control vector or miR-99b-let-7e-miR-125a (cluster) in addition to GFP. Contribution to PB by control or cluster-transduced BM cells (GFP+) at indicated time is shown. The first time point indicates GFP+% in the culture 2 days post-transduction. wks: weeks; mths: months. \* p < 0.05. **(D)** Multi-lineage differentiation into

myeloid (Mac-1+), B (B220+) and T lineages (CD3+) among GFP+ cells 5 months post-transplantation. C-D: n = 4-7 each. Error bars indicate standard deviation.

### **Figure 3. MiR-125a Enhances Hematopoietic Stem Cell Function.**

The miR-99b-let-7e-miR-125a cluster or individual miRNA was transduced and transplanted as in Figure 2C. PB contribution by transduced BM cells was analyzed 4 months post-transplantation. **(A)** Representative FACS plots. **(B)** Quantification of data shown in (A). **(C)** Comparison of contribution to blood formation by control or miR-125a alone (125a). The first time point (3 days) indicates GFP+% in the culture post-transduction. **(D)** Multi-lineage differentiation by transduced BM cells. For B-D: n = 1-5 per group per time point shown. **(E)** Contribution to blood formation by transduced BM cells that were cultured *ex vivo* for 18 days. n = 4 for control (Ctrl) and n = 5 for miR-125a (125a). **(F)** Serial methylcellulose colony formation in the presence of a control or miR-125a-specific antagomir. Representative fields from primary ( $1^0$ ) and secondary ( $2^0$ ) cultures. **(G-H)** Purified HSPCs were transduced with control or miR-125a and transplanted. PB contribution in recipients was quantified. See Supporting Information for population definition. Each animal received either (H) 100 SLAM, (G) 1,000 LKS or 10,000 progenitors together with  $2.5 \times 10^5$  supporting BM cells. n = 5 except for miR-125a-transduced CMP (n = 4). Error bars reflect standard deviation.

### **Figure 4. MiR-125a Inhibits Immature Hematopoietic Cell Apoptosis and Targets Bak1**

**(A)** Selective protection against apoptosis by miR-125a in lineage negative cells. BM cells were analyzed for lineage markers (Lin), AnnexinV and 7-AAD with flow cytometry. AnnexinV histograms show Lin- and Lin+ populations after gating for 7-AAD negative cells. Lin- population is defined as the lowest 3% cells expressing lineage markers. n=5.

**(B)** Quantification of data in (A). Percentage of AnnexinV+ (AV+) cells present in 7AAD-Lin-GFP+ cells are shown. n = 5. \* p < 0.05. **(C)** HL-60 cells were transduced with miR-125a or a control vector (Ctrl). Western blot was probed for Bak1 and  $\beta$ -actin. **(D)** Luciferase reporters of WT (wide-type) or Mut (mutant for miR-125a site) Bak1 3'UTR was analyzed in the presence of miR-125a or a control vector in 293T cells. Normalized luciferase activities are shown. Error bars represent standard deviation. **(E-F)** 5FU primed wild type donor marrow was co-transduced with miR-125a (DsRedExpress+) and a virus for either control or Bak1 (GFP+). Cells were (E) cultured *in vitro* for 6 days or (F) transplanted (n=4) and analyzed 4 weeks afterwards. The percentage of co-transduced cells (GFP+DsRedExpress+) in culture or in PB is shown.

## TABLE LEGENDS

Total GFP+ bone marrow cells were FACS-sorted from primary recipients 4-5 months post-transplantation and transplanted into secondary recipients together with 200,000 un-fractionated wild-type bone marrow cells at indicated cell doses. The number of GFP+ HSCs per two legs in each donor animal was calculated by multiplying GFP+ HSC frequency, donor GFP+% and donor BM cellularity.

\* Positive responder called if  $\geq 1\%$  GFP+ cells present in myeloid (Mac1+) and lymphoid (B220+ or CD3+) lineages 16 weeks post transplantation.

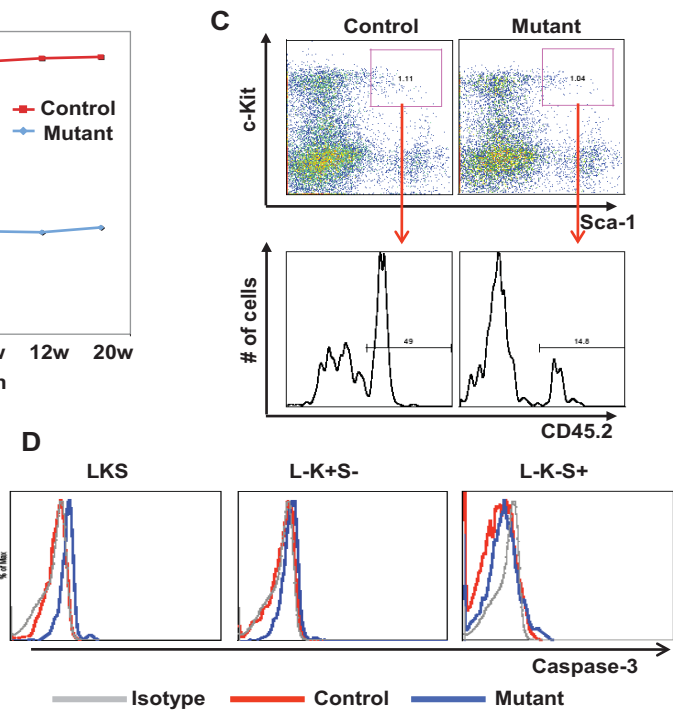
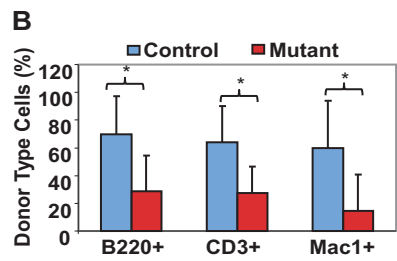
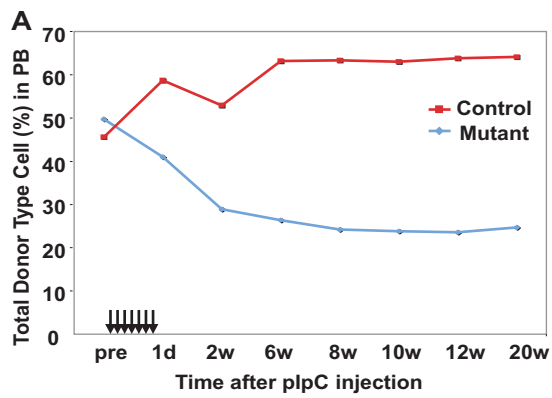
† Bone marrows from 3 control mice were pooled to yield enough donor cells.

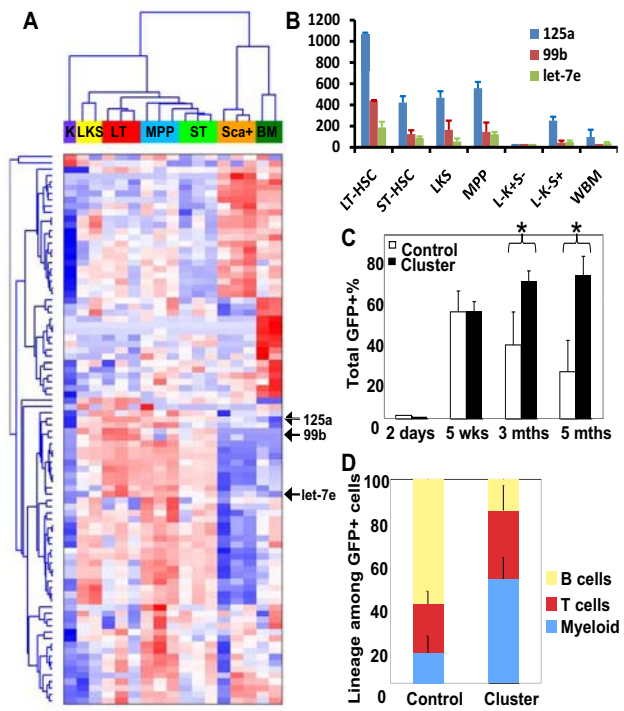
‡ Calculated with L-Calc.

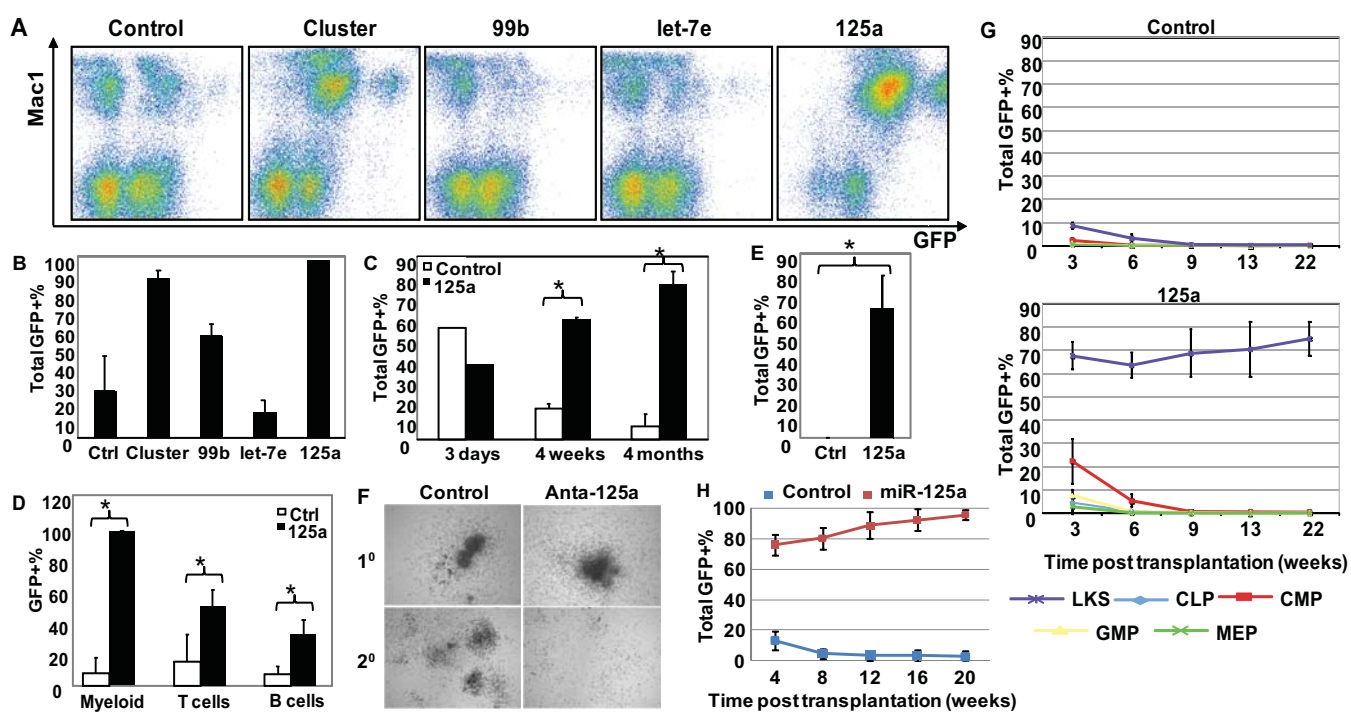
§ p = 0.02.

¶ p = 0.0001.

|| Certain animals were lost due to accidental dehydration. Initially 10 animals in group.









**Table 1. Experimental scheme and positive responders in two independent limiting dilution assay experiments.**

	Experiment 1						Experiment 2					
	Donor GFP+ %	Donor BM Cellularity (2 legs)	Cell doses (x10 <sup>5</sup> )	Positive responders*/ total scored animals	GFP+ HSC frequency <sup>‡</sup> ( x10 <sup>-6</sup> )	GFP+ HSC numbers (2 legs)	Donor GFP+ % <sup>†</sup>	Donor BM Cellularity (2 legs)	Cell doses (x10 <sup>5</sup> )	Positive responders*/ total scored animals	GFP+ HSC frequency <sup>‡</sup> ( x10 <sup>-6</sup> )	GFP+ HSC numbers (2 legs)
Control	35.6%	119 x10 <sup>6</sup>	20	4/6 <sup>  </sup>	1 in 2.1 <sup>§</sup>	20.2	16.6%	127 x10 <sup>6</sup>	25	6/10	1 in 2.4 <sup>¶</sup>	8.8
			2	0/2 <sup>  </sup>			17.7%	183 x10 <sup>6</sup>	5	3/10		13.5
			0.5	0/10			8.4%	96 x10 <sup>6</sup>	1	0/10		3.4
			0.1	0/10			0.3	0/9				
			20	9/9			25	10/10				
miR-125a	86.5%	94 x10 <sup>6</sup>	2	2/10	1 in 0.5 <sup>§</sup>	162.6	78.1%	177 x10 <sup>6</sup>	5	9/10	1 in 0.18 <sup>¶</sup>	768
			0.5	2/10					1	4/10		
			0.1	0/10					0.3	3/10		
			20	9/9					25	10/10		
			2	2/10					5	9/10		
GFP+ HSC expansion	8.1 X						56.9 – 225.9 X					

